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Research Article

Quantitative Proteomics Analysis by Isobaric Tags for Relative and Absolute Quantitation Identified Lumican as a Potential Marker for Acute Aortic Dissection

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Acute aortic dissection (AAD) is a serious vascular disease. Currently the diagnosis relies on clinical and radiological means whereas serum biomarkers are lacking. The purpose of this study was to identify potential serum biomarkers for AAD using isobaric tags for relative and absolute quantitation (iTRAQ) approach. A total of 120 serum samples were collected from three groups: AAD patients ($n = 60$), patients with acute myocardial infarction (AMI, $n = 30$), and healthy volunteers ($n = 30$), whereas the first 10 samples from each group were used for iTRAQ analysis. Using iTRAQ approach, a total of 174 proteins were identified as significantly different between AAD patients and healthy subjects. Among them, forty-six proteins increased more than twofold, full-scale analysis using serum sample for the entire 120 subjects demonstrated that Lumican level was significantly increased relative to control and AMI samples. Further, Lumican level correlated with time from onset to admission in AAD but not AMI samples. Using iTRAQ approach, our study showed that Lumican may be a potential AAD-related serum marker that may assist the diagnosis of AAD.

1. Introduction

Acute aortic dissection (AAD) has become a treatable disease due to recent advances in new therapeutic approaches for the management of heart and arterial diseases; however, development of quick and economic diagnostic methods remains a challenge. Variability in disease presentation often obscures diagnoses, and imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), and esophagus ultrasound remain prohibitive due to cost and availability. Aortic dissection remains a frequent target of medicolegal litigations with accusations of failure to diagnose against treating physicians and hospitals [1]. Some progress in the biochemical diagnosis of AAD has been made in the last decade [2, 3]; several acute phase proteins and coagulation parameters were identified to increase in AAD patients, but these are nonspecific biomarkers for AAD as they may be also aberrantly expressed in other disease conditions such as acute myocardial infarction (AMI).

Recently a quantitative proteomic assay, isobaric tags for relative and absolute quantitation (iTRAQ), has been developed and utilized to identify biomarkers for various disease conditions [4, 5]. This chemical labeling method involves the stable incorporation of isotopes into an amine tagging reagent, which can then be reliably detected by mass spectrometry, thereby permitting comparative quantitation of various proteins in a multiplex manner. It has been suggested to be suitable for the discovery of biomarkers in a wide range of body fluids and tissues, including serum and plasma [5, 6]. With this method, we expect to find the potential biomarkers which are released from the disruption of the aortic media and can provide sufficient specificity and longer time window for the diagnosis of AAD.

2. Materials and Methods

2.1. Samples. The study included a total of 30 healthy individuals and 90 patients (60 AAD, 30 AMI). All the patients were selected in a consecutive manner from the period of July 2009 to November 2011 from Fudan University affiliated Zhongshan Hospital (Shanghai, China). iTRAQ analysis was performed for the first twenty patients (10 AAD and 10 AMI) and ten healthy individuals. All patients presented within 72 hours after an episode of chest and/or back pain lasting 5 minutes or more. The diagnosis of AAD was confirmed by computed tomographic arteriography (CTA). The AMI patient was confirmed by electrocardiography (ECG) and cardiac troponin T (cTNT) tests. All patients gave their informed consent for the study. The protocol was approved by the Ethics Committee of Zhongshan Hospital.

For each study subject, whole blood samples were immediately collected in BD Vacutainer SST tubes (BD Diagnostics, Plymouth, UK) after admission and centrifuged at 4000 rpm for 10 min at room temperature. The serum was frozen and stored in aliquots at −80°C until analysis.

2.2. Serum C-Reactive Protein and Myoglobin Test. Vitros 5.1 FS automatic biochemistry analyzer (Johnson & Johnson; Calif, USA) was used for serum C-reactive protein (CRP) test, and Cobas e411 immunoassay analyzer (Roche; Mannheim, Germany) was used for the serum myoglobin (Myo) test. The results were then interpreted in accordance with that tested by the International Federation of Clinical Chemistry (IFCC) recommended method. Analyses were performed immediately after the centrifugation of whole blood samples.

2.3. iTRAQ Sample Preparation: Strong Cation Exchange (SCX) Chromatography. iTRAQ reagents were purchased from Applied Biosystems (Foster City, USA). Fourteen interfering highly abundant proteins from serum samples were removed using Agilent multiple affinity removal liquid chromatography (LC) column-Human 14 (MARS) (shimadzu, Kyoto, Japan). One hundred micrograms of each extract were precipitated using acetone at −20°C and suspended in 20 μL of dissolution buffer (Applied Biosystems, Foster City, USA). After reduction and alkylation, each sample was digested with trypsin (w(trypsin) : w(protein) = 1 : 20) at 37°C overnight. The tryptic peptides were labeled with the iTRAQ reagents as follows: normal controls group was labeled with iTRAQ 113, AMI group was labeled with iTRAQ 114, and AAD group was labeled with iTRAQ 115. The peptides were pooled and desalted with Sep-Pak Vac C18 (Waters, Milford, USA). The peptide mixture was diluted with buffer A containing 10 mM KH_2PO_4 in 25% acetonitrile (ACN) at pH 2.6. The peptides were fractionated by 20AD high-performance liquid chromatography (HPLC) system (Shimadzu; Kyoto, Japan) equipped with a polysulfoethyl A column (2.1 mm × 100 mm, 5u, 200 A; The Nest Group, Southborough, Mass). The composition of buffer B was 350 mM KCl, 10 mM KH_2PO_4, and 25% ACN at pH 2.6. Separation was performed using a linear binary gradient of 0–80% buffer B in buffer A at a flow rate of 200 μL/min for 60 min. The fractions were combined into 20 groups.

2.4. LC-MS Analysis. Each SCX fraction was dried down by the rotary vacuum concentrator, dissolved in buffer C (0.1% formic acid, 5% ACN, 95% water), and analyzed on Qstar XL (Applied Biosystems; Foster City, USA). The HPLC gradient was 5–35% buffer D (95% ACN, 0.1% formic acid) in buffer C at a flow rate of 300 nL/min for 70 min. Analysis survey scans were acquired MS from m/z 400–1800 with up to 4 precursors selected for MS/MS from m/z 100–2000.

2.5. The Confirmative ELISA Analysis for Lumican. The confirmative ELISA analysis for Lumican was performed using the kits from CUSABIO BIOTECH CO, following manufacture's recommendation (CUSABIO BIOTECH CO., LTD., Wuhan, China).

2.6. Data Analysis. All statistical analyses were performed in SPSS 12.0 (SPSS Inc. Chicago, USA). Results were presented as Mean ± SD. A comparative analysis of multiple groups was performed with a one-way-ANOVA or Mann-Whitney/Kruskal-Wallis Test. Statistical significance was defined as $P < 0.05$. Peptide and protein identification was performed by searching the MS/MS spectra against the SwissProt database using the local Protein Pilot 2.0.1 software. Only peptides identified with confidence interval values of no less than 95% (Unused ProtScore >1.3) were used for protein identification compilation and subsequent quantitation calculation. Fold changes of >2 or <0.5 were set as cut-off values to designate significant differences in protein expression among the AAD group and the normal control group.

2.7. PANTHER Analysis. The PANTHER database was used to elucidate cellular components, biological processes, and the molecular functions associated with each individual protein (http://www.pantherdb.org/).

3. Results

3.1. Clinical Features of Study Subjects. The clinical features of the AAD patients, AMI patients, and normal controls are summarized in Table 1. There were no differences in age distribution and sex composition among the three groups involved either for ELISA analysis ($N = 120$) ($P = 0.351$ and 0.378, resp.,) or iTRAQ analysis ($P = 0.241$ and 0.873, resp.). There was no differences in the time from onset to admission between AAD and AMI group either ($P = 0.776$).

3.2. Functional Classification of Identified Proteins by iTRAQ. A total of 174 proteins with confidence interval values of no less than 95% were identified (Unused ProtScore > 1.3). However, after manually rechecking the MS/MS data thoroughly peak by peak, 155 proteins (89.08%) had a relative quantitation of one or more peptides. Fifteen proteins had no quantifiable peptides that could be ascertained, and four

Table 1: Clinical characteristics in three groups.

		AAD	AMI	Normal controls	*P* value
	n	60	30	30	
ELISA test ($N = 120$)	Age (Mean ± SD)	55.63 ± 16.39	59.70 ± 13.98	59.50 ± 12.65	0.351[a]
	Gender, *n* (%), male	30 (50)	17 (56.67)	16 (53.33)	0.378[b]
	Admission after onset hours (Mean ± SD)	20.19 ± 18.09	19.33 ± 15.31	/	0.776[c]
	Type A *n* (%)	31 (51.67)	/	/	
	Type B *n* (%)	29 (48.33)	/	/	
	Marfan *n* (%)	7 (11.67)	/	/	
	n	10	10	10	
iTRAQ test ($N = 30$)	Age (Mean ± SD)	51.60 ± 13.22	61.00 ± 5.25	49.9 ± 15.02	0.241[a]
	Gender, *n* (%), male	6 (60)	6 (60)	5 (50)	0.873[b]
	Admission after onset hours (Mean ± SD)	27.20 ± 24.56	18.40 ± 23.12	/	0.363[c]
	Type A *n* (%)	5 (50)	/	/	
	Type B *n* (%)	5 (50)	/	/	
	Marfan *n* (%)	2 (20)	/	/	

[a]One-way-ANOVA; [b]Chi-square Test; [c]Mann-Whitney Test.

proteins had peptides with confidence interval values that were less than 95%.

In total, 174 proteins were sorted using the PANTHER classification system, which sorts the proteins into respective categories based on their molecular functions. The major groups include: signaling molecules (13%), enzyme modulators (12%), transfer/carrier proteins (11%), and proteases (10%). Other groups include: structural proteins (1%), cell adhesion molecules (1%), cytoskeletal proteins (3%), extracellular matrix proteins (2%), and cell junction proteins (1%).

As a way to cross-check the reliability of quantitation of iTRAQ reagent, serum CRP and Myo levels were assessed using both conventional biochemical and immunoassay tests and iTRAQ analysis on the same specimens. With biochemical and immunoassay analysis, CRP was 41.31 ± 32.76 mg/mL and Myo was 66.42 ± 81.23 mg/mL in AAD group, while in normal controls, the former was 5.88 ± 1.42 and the latter was 32.07 ± 14.14 mg/mL. CRP and Myo levels of AAD patients were 7.03-fold and 2.07-fold higher, respectively, than normal controls. Using iTRAQ, the AAD/normal controls ratios of CRP and Myo were similar at 9.12-fold (Table 2) and 1.47-fold. The ratios of CRP and Myo among three groups were similar with either biochemical and immunoassay or iTRAQ analysis, confirming the reliability of iTRAQ analysis.

3.3. Proteins with Over Twofold Differential Expression. A total of 155 proteins had a relative quantitation difference for AAD patients compared with the normal control group of which 46 proteins increased more than twofold (Table 2), while 36 proteins decreased more than twofold among the AAD patients (Table 3). Among the identified proteins with increased levels in AAD, there were a number of acute phase reactants (CRP, Beta-2-microglobulin, Complement factor I), blood coagulation marker (Haptoglobin, Coagulation factor V, Coagulation factor IX), and cellular components (Lumican, Tubulin beta-3 chain, Fibronectin). However when compared to AMI patients, 14 of the 46 protein showed less than 2-fold increase, including complement component 9, complement factor1, Plasma protein C1 inhibitor, and Ig Kappa chain C-region (Table 2). Interestingly, some acute phase proteins such as CRP remains on the list as it showed the differential expression between the two conditions.

Among proteins with decreased expression in AAD patients compared with normal controls, there were a number of molecules involved in protein metabolism (Inter-alpha-trypsin inhibitor heavy chain H2, Alpha-2-HS-glycoprotein), lipid metabolic process (Apolipoprotein A-IV, Apolipoprotein E, Apolipoprotein C-I), blood coagulation marker (Fibrinogen alpha chain, Prothrombin), and cellular components (Alpha-2-HS-glycoprotein, thrombospondin-1 (TSP-1)). When compared to AMI patients, 8 of 36 proteins did not reach the 2-fold differential expression (Table 3).

3.4. The ELISA Analysis of Serum Concentrations of Lumican. Based on the iTRAQ findings above we selected two targets, Fibronectin and Lumican, the protein markers potentially associated with vascular injury, for the validation using ELISA method. At the initial analysis using 10 AAD and 10 normal samples we found that statistical significant difference between AAD and normal individual was seen for

Table 2: List of the increased (>2-fold) protein targets identified and their corresponding class, associated biological process, and cellular component.

N	Unused[a]	Peptides[b]	Accession #	Name	Biological process	Cellular component	Protein class	AAD/CON ratio	AAD/AMI ratio
1	2.7	1	Q13509	Tubulin beta-3 chain	Cellular component morphogenesis	Cytoskelelton	Cytoskeletal protein/tubulin	39.8406	0.0711
2	2	1	P01600	Ig kappa chain V-I region Hau			Unclassified	31.3480	3.0760
3	6.67	3	P02743	Serum amyloid P-component	Response to stress		Defense/immunity protein/antibacterial response	24.4499	9.1241
4	13.53	10	P05546	Heparin cofactor 2	Protein metabolic process		Enzyme modulator	19.7628	11.2740
5	17.67	9	P36955	Pigment epithelium-derived factor	Protein metabolic process		Enzyme modulator	19.7628	9.8135
6	6.24	4	P05543	Thyroxine-binding globulin	Protein metabolic process		Enzyme modulator	12.7065	3.7665
7	12.63	18	P01834	Ig kappa chain C region	Response to stimulus	Immunoglobulin complex	Defense/immunity protein	11.6959	1.8031
8	55.14	43	P02751	Fibronectin	Blood coagulation	Extracellular matrix	Transfer/carrier protein	11.5875	3.7327
9	35.94	29	P01011	Alpha-1-antichymotrypsin	Protein metabolic process		Enzyme modulator	10.5708	8.0906
10	2.22	1	P02741	C-reactive protein	Response to stress		Defense/immunity protein	9.1241	5.9701
11	2	1	Q9UK55	Protein Z-dependent protease inhibitor	Protein metabolic process		Enzyme modulator	8.7873	2.4888
12	100.78	51	P04114	Apolipoprotein B-100	Lipid metabolic process		Transfer/carrier protein	8.6281	2.8050
13	9.19	4	P35858	Insulin-like growth factor-binding protein complex acid labile chain	Cell-cell adhesion	Extracellular matrix	Receptor	8.4746	4.0177
14	47.88	42	P19827	Inter-alpha-trypsin inhibitor heavy chain H1	Protein metabolic process		Enzyme modulator	5.8072	5.6497
15	106.72	89	P00450	Ceruloplasmin	Blood coagulation	Extracellular matrix	Transporter	5.1046	9.6339
16	37.56	23	P04196	Histidine-rich glycoprotein	Blood coagulation		Unclassified	4.6125	3.2206
17	4.15	2	P08571	Monocyte differentiation antigen CD14	Immune system process		Receptor	4.0933	2.4888
18	2.72	1	Q96KN2	Beta-Ala-His dipeptidase	Protein metabolic process		Protease	4.0933	1.9231
19	31.66	20	P01871	Ig mu chain C region	Response to stimulus		Defense/immunity protein	3.8023	2.3552

Table 2: Continued.

N	Unused[a]	Peptides[b]	Accession #	Name	Biological process	Cellular component	Protein class	AAD/CON ratio	AAD/AMI ratio
20	12.12	6	P51884	Lumican	Cell-cell adhesion	Extracellular matrix	Receptor	3.6311	1.2942
21	2.71	1	P00740	Coagulation factor IX	Blood coagulation		Protease	3.4037	2.0137
22	6.27	3	P08185	Corticosteroid-binding globulin	Protein metabolic process		Enzyme modulator	3.2808	5.4945
23	2.58	21	P00739	Haptoglobin-related protein			Unclassified	3.2206	1.7538
24	4.98	4	P04003	C4b-binding protein alpha chain	Blood coagulation		Transfer/carrier protein	3.1626	5.0582
25	6.02	6	P02745	Complement C1q subcomponent subunit A	Response to stimulus		Transfer/carrier protein	3.1046	3.6982
26	5.83	3	P22792	Carboxypeptidase N subunit 2	Cell adhesion	Extracellular matrix	Receptor	3.0202	1.3805
27	15.99	11	P05155	Plasma protease C1 inhibitor	Protein metabolic process		Enzyme modulator	2.9647	1.2824
28	2.02	2	P01742	Ig heavy chain V-I region EU			Unclassified	2.8580	1.1695
29	2	1	P09486	SPARC	Cell-cell signaling		Transfer/carrier protein	2.7042	0.7312
30	18.06	9	P02760	Protein AMBP	Blood coagulation		Enzyme modulator	2.6062	3.5651
31	8.99	5	P12259	Coagulation factor V	Blood coagulation	Extracellular matrix	Transporter	2.6062	3.2206
32	8.31	6	P27169	Serum paraoxonase/arylesterase 1	Immune system process		Oxidoreductase	2.5349	5.6497
33	2.32	1	P19320	Vascular cell adhesion protein 1	Cell-cell adhesion		Defense/immunity protein	2.3121	2.1478
34	2.01	1	O00187	Mannan-binding lectin serine protease 2	Response to stimulus		Protease	2.3121	1.5277
35	43.19	29	P02749	Beta-2-glycoprotein 1	Blood coagulation		Transfer/carrier protein	2.2287	7.1124
36	3.52	2	P11226	Mannose-binding protein C	Response to stimulus		Defense/immunity protein	2.2080	2.6062
37	87.93	67	P00738	Haptoglobin	Blood coagulation		Protease	2.1678	3.5651
38	5.7	4	Q13790	Apolipoprotein F	Lipid metabolic process		Transporter	2.1478	3.9448
39	65.42	41	P02790	Hemopexin	Vitamin transport		Transfer/carrier protein	2.1478	3.9078
40	2.96	1	P20851	C4b-binding protein beta chain	Blood coagulation		Transfer/carrier protein	2.1281	4.8309

Table 2: Continued.

N	Unused[a]	Peptides[b]	Accession #	Name	Biological process	Cellular component	Protein class	AAD/CON ratio	AAD/AMI ratio
41	2	1	P61769	Beta-2-microglobulin	Response to stimulus		Defense/immunity protein	2.1088	2.1281
42	20.86	10	P02748	Complement component C9	Response to stimulus		Receptor	2.1088	0.6252
43	6.96	3	P07477	Trypsin-1	Protein metabolic process		Protease	2.1088	0.3436
44	13.64	7	P05156	Complement factor I	Response to stimulus		Protease	2.0325	0.9120
45	15.17	10	P01842	Ig lambda chain C regions	Response to stimulus	Immunoglobulin complex	Defense/immunity protein	2.0137	2.1678
46	4.29	1	P35030	Trypsin-3	Protein metabolic process		Protease	2.0137	1.3931

[a]Unused > 1.3 means at least 95% confidence; [b]number of peptides with 95% confidence; AAD: acute aortic dissection; AMI: acute myocardial infarction; CON: normal controls.

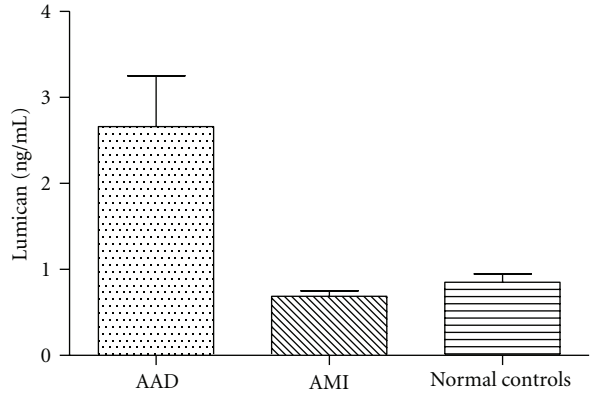

Figure 1: Lumican levels were significant difference between AAD, AMI, and normal individuals (Mean ± SEM; $P = 0.003$).

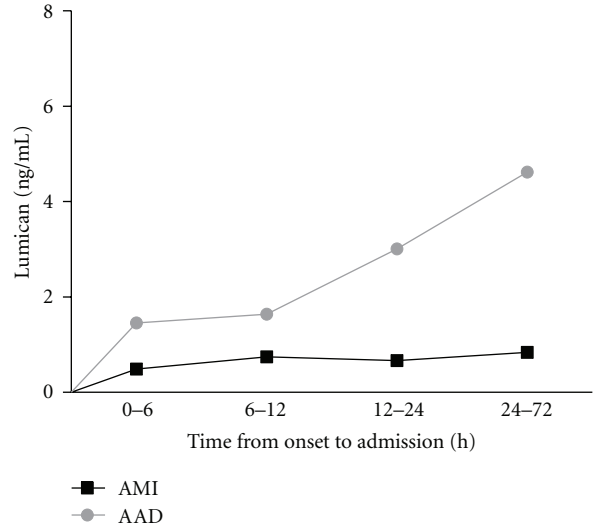

Figure 2: Lumican levels were correlation with time from onset to admission in AAD group ($r = 0.256$, $P = 0.048$), but not in AMI group ($r = 0.077$, $P = 0.685$).

Lumican but not Fibronectin (data not shown). Therefore, we carried a full validation study only for Lumican, using the entire 120 samples collected (see Table 1). A statistical significant difference between AAD, AMI, and normal individual was seen in serum concentrations of Lumican (2.66 ± 4.58 ng/mL in AAD group, 0.69 ± 0.34 ng/mL in AMI group, and 0.85 ± 0.53 ng/mL in normal control, $P = 0.003$). The difference for AAD and AMI also reached statistical significance ($P < 0.05$) suggesting the specificity of this marker for AAD (Figure 1). We further analyzed the correlation between Lumican levels with time from onset of symptoms to admission. As shown in Figure 2, a correlation was seen in AAD group ($r = 0.256$, $P = 0.048$) but not in AMI group ($r = 0.077$, $P = 0.685$), further confirming the specificity of Lumican as a marker for AAD.

4. Discussion

iTRAQ analysis is recently been used as a potentially more effective biomarker discovery method than traditional proteomic methods. The high reproducibility optimizes this technique for embarking on "fishing-expeditions" as an initial screening for potential useful biomarkers [6–8]. As a means of internal validation, the iTRAQ method was compared with CRP biochemical assay and Myo immunoassay. In our study there were no significant differences in the serum levels determined by the different methods. Thus, the iTRAQ method we employed appears in this preliminary analysis to be suitable for the detection of relevant proteins.

To identify differentially expressed proteins, in many studies, the cut-off points were set at 20% to 50% average variance [7, 9, 10]. However, such approaches may result in finding markers with low specificity [2, 3]. We therefore appropriated to increase the cut-off point at 100% variance

Table 3: List of the decreased (<0.5 folds) protein targets identified and their corresponding class, associated biological process, and cellular component.

N	Unused[a]	Peptides[b]	Accession #	Name	Biological process	Cellular component	Protein classification	AAD/CON ratio	AAD/AMI ratio
1	19.31	17	P02775	Platelet basic protein	Blood coagulation		Transfer/carrier protein	0.0209	0.0398
2	44.6	31	P02671	Fibrinogen alpha chain	Blood coagulation	Extracellular matrix	Transfer/carrier protein	0.0370	0.0203
3	19.82	30	P02656	Apolipoprotein C-III	Lipid metabolic process		Transporter	0.0570	0.0240
4	5.7	5	P01717	Ig lambda chain V-IV region Hil			Unclassified	0.0655	0.0679
5	8.45	4	P02768	Serum albumin	Transport		Transfer/carrier protein	0.0724	0.8472
6	3.22	1	P04264	Keratin, type II cytoskeletal 1	Cellular component morphogenesis	Cytoskelelton	Structural protein	0.0780	0.9639
7	2.05	1	P04070	Vitamin K-dependent protein C	Blood coagulation		Protease	0.0794	0.0441
8	34.98	27	P02652	Apolipoprotein A-II	Lipid metabolic process		Transporter	0.0991	0.1067
9	20.3	10	P02654	Apolipoprotein C-I	Lipid metabolic process		Transporter	0.1159	0.0847
10	94.94	108	P02765	Alpha-2-HS-glycoprotein	Protein metabolic process	Extracellular matrix	Extracellular matrix protein	0.1180	0.1472
11	39.07	25	P01008	Antithrombin-III	Protein metabolic process		Enzyme modulator	0.1202	0.0973
12	72.69	57	P00734	Prothrombin	Blood coagulation		Enzyme modulator	0.1225	0.2051
13	6.88	3	P27918	Properdin	Response to stimulus		Unclassified	0.1419	0.1905
14	6.31	4	P69905	Hemoglobin subunit alpha	Blood circulation		Transfer/carrier protein	0.1500	0.2312
15	8.97	15	Q03591	Complement factor H-related protein 1	Blood coagulation		Transfer/carrier protein	0.1706	0.0991
16	27.03	22	P10909	Clusterin	Apoptosis		Unclassified	0.1905	0.1076
17	2	1	Q15942	Zyxin	Cellular component morphogenesis		Enzyme modulator	0.1923	0.2109
18	20.4	26	P01024	Complement C3	Protein metabolic process		Transfer/carrier protein	0.2070	0.1406
19	7.43	3	P17936	Insulin-like growth factor-binding protein 3	Cell-matrix adhesion		Unclassified	0.2089	0.3162
20	2.23	2	P55290	Cadherin-13	Cell-cell adhesion	Cell junction	Receptor	0.2188	0.1660
21	1.41	1	P13598	Intercellular adhesion molecule 2	Cell-cell adhesion		Transfer/carrier protein	0.2355	0.1600

Table 3: Continued.

N	Unused[a]	Peptides[b]	Accession #	Name	Biological process	Cellular component	Protein classification	AAD/CON ratio	AAD/AMI ratio
22	96.24	64	P00751	Complement factor B	Blood coagulation		Transfer/carrier protein	0.2466	0.2729
23	28.82	16	P09871	Complement C1s subcomponent	Blood coagulation		Protease	0.2606	0.4169
24	13.74	9	P01019	Angiotensinogen	Protein metabolic process		Enzyme modulator	0.2630	0.5058
25	95.34	74	P19823	Inter-alpha-trypsin inhibitor heavy chain H2	Protein metabolic process		Enzyme modulator	0.2805	0.5649
26	5.15	2	P18065	Insulin-like growth factor-binding protein 2	Cell-matrix adhesion		Unclassified	0.2884	0.3281
27	16.84	10	P07996	Thrombospondin-1	Blood coagulation	Extracellular matrix	Transfer/carrier protein	0.3404	0.8317
28	5.31	3	P26927	Hepatocyte growth factor-like protein	Blood coagulation		Transfer/carrier protein	0.3436	0.3436
29	7.85	4	O14791	Apolipoprotein L1	Lipid metabolic process		Transporter	0.3908	0.2831
30	94.76	57	P06727	Apolipoprotein A-IV	Lipid metabolic process		Transporter	0.4018	0.1837
31	14.1	9	P35527	Keratin, type I cytoskeletal 9	Cellular component morphogenesis		Structural protein	0.4055	0.7244
32	9.48	6	P00746	Complement factor D	Blood coagulation		Protease	0.4571	0.6668
33	48.09	24	P02649	Apolipoprotein E	Lipid metabolic process		Transporter	0.4656	0.2051
34	38.8	32	P02735	Serum amyloid A protein	Immune system process		Transporter	0.4742	0.0319
35	9.46	5	P10720	Platelet factor 4 variant	Blood coagulation		Transfer/carrier protein	0.4742	0.5105
36	53.31	29	P02774	Vitamin D-binding protein	Transport		Transfer/carrier protein	0.4831	2.2287

[a]Unused > 1.3 means at least 95% confidence; [b]number of peptides with 95% confidence; AAD: acute aortic dissection; AMI: acute myocardial infarction; CON: normal control.

in serum levels of candidate proteins between AAD patients and normal subjects. Thus, only twofold changes below or above normal controls were considered significant. In our study, total of 155 proteins had a relative difference between AAD patients and healthy volunteers. Therefore, with higher specificity, these candidate proteins are more likely to be potential biomarkers for AAD.

In the group of significantly increased proteins, there were numerous acute phase reactants, such as Beta-2-microglobulin (P61769), which could be indicative of an increased inflammatory response among AAD patients. CRP (P02741), a protein found to be elevated in patients who presented with symptoms or rupture of AAD and abdominal aortic aneurysm, was also identified using iTRAQ [11, 12]. CRP is a nonspecific biomarker associated with AAD and a predictor for long-term adverse events [13], and it can be used to monitor evolution of false lumen thrombosis [14]. Unfortunately, CRP is also produced in coronary plaques [15], acute myocardial infarction [16], and so forth. The elevations of these acute phase reactants represent a generalized reaction to vascular injury, and as such, they are nonspecific biomarkers. In addition, many proteins identified are associated with blood coagulation and fibrinolytic system. Among which, ten had increased serum levels

(e.g., P00450-Ceruloplasmin, P02751-Fibronectin, P00738-Haptoglobin...), and twelve had decreased serum levels (e.g., P02671-Fibrinogen alpha chain, P00751-Complement factor B, P00734-Prothrombin...). The pathophysiological mechanism for the appearance of these proteins may be explained by the release of tissue factors from the dissected aortic wall then the activation of the extrinsic coagulation system [17–19]. In addition, platelets can be activated by injuries to the vessel wall, activation of the coagulation cascade, or by activating factors released from stimulated endothelial cells and platelets (e.g., ADP, thromboxane, von Willebrand Factor). It also has been found that platelet functions were affected secondary to acute massive consumption coagulopathy in the false lumen in AAD patient [20, 21].

In the past few years, extracellular matrix (ECM) components of vessel walls such as elastin have been shown to be elevated in aortic dissections; however, such increases were less than twofold [3]. Our study found 9 extracellular matrix component proteins with greater than twofold differences, among these are Carboxypeptidase (P22792), Lumican (P51884), Fibronectin (P02751), Ceruloplasmin (P00450), and Thrombospondin-1 (TSP-1, P07996). Fibronectin is a polymorphic and multifunctional glycoprotein that plays wide-ranging roles in tissue injury [22–25]. TSP-1, which is an extracellular protein that participates in cell-to-cell and cell-to-matrix communication, can stimulate or inhibit the migration of vascular smooth muscle cells or endothelial cells. It has been known as a plasma marker of peripheral arterial disease [26].

Lumican is distributed in interstitial collagenous matrices throughout the body. In coronary arteries ischemic lesion, it is overexpressed by vascular smooth muscle cells (VSMCs) [27] and also synthesized in aortic smooth muscle cells [28]. In iTRAQ analysis, serum Lumican levels in patients with AAD were 1.29-fold and 3.63-fold higher than in patients with AMI and normal controls, respectively. It is interesting to note that with iTRAQ analysis, the level of difference between AAD and AMI for Lumican is less than that of Fibronectin (Table 2), yet the initial validation using ELISA method showed that only Lumican was significantly increased in AAD and AMI samples. While there may be variety reasons to explain the variations of the findings between the two methods, it highlights the importance of validation in biomarker studies. The finding that Lumican expression correlated with the time from onset to admission only in AAD but not in AMI sample further confirmed the specificity of this protein in association with AAD.

Proteomic approach provides an exciting platform to identify clinically useful protein biomarkers. As an initial step our study identified potential candidate protein biomarkers in the serum of AAD patients with the iTRAQ technique. However, the ultimate development of biomarkers which provide sufficient sensitivity or specificity for the diagnosis of AAD will require multiple validations and clinical testing, which may include nonprotein markers. Nevertheless our findings provide preliminary list of candidate biomarkers that should be further validated, either alone or in combination.

5. Conclusion

In this paper, we found that iTRAQ technique is a suitable approach for the detection of the new potential protein markers in the serum of AAD patients. Using iTRAQ approach, our study identified that Lumican may be a potentially interesting new serum marker of AAD, and upon further validation this marker may assist the clinical diagnosis of AAD.

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